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Evaluating the Effects of BPA and TBBPA Exposure on Pregnancy Loss and Maternal–Fetal Immune Cells in Mice

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Background: Bisphenol A (BPA) exposure has been linked to miscarriages and pregnancy complications in humans. In contrast, the potential reproductive toxicity of BPA analogs, including tetrabromobisphenol A (TBBPA), is understudied. Furthermore, although environmental exposure has been linked to altered immune mediators, the effects of BPA and TBBPA on maternal–fetal immune tolerance during pregnancy have not been studied. The present study investigated whether exposure resulted in higher rates of pregnancy loss in mice, lower number of regulatory T cells (Tregs), and lower indoleamine 2,3 deoxygenase 1 (*Ido1*) expression, which provided evidence for mechanisms related to immune tolerance in pregnancy.

Objectives: The purpose of this investigation was to characterize the effects of BPA and TBBPA exposure on pregnancy loss in mice and to study the percentage and number of Tregs and *Ido1* expression and DNA methylation.

Methods: Analysis of fetal resorption and quantification of maternal and fetal immune cells by flow cytometry were performed in allogeneic and syngeneic pregnancies. *Ido1* mRNA and protein expression, and DNA methylation in placentas from control and BPA- and TBBPA-exposed mice were analyzed using real-time quantitative polymerase chain reaction, immunofluorescence, and bisulfite sequencing analyses.

Results: BPA and TBBPA exposure resulted in higher rates of hemorrhaging in early allogeneic, but not syngeneic, conceptuses. In allogeneic pregnancies, BPA and TBBPA exposure was associated with higher fetal resorption rates and lower maternal Treg number. Importantly, these differences were associated with lower IDO1 protein expression in trophoblast giant cells and higher mean percentage *Ido1* DNA methylation in embryonic day 9.5 placentas from BPA- and TBBPA-exposed mice.

Discussion: BPA- and TBBPA-induced pregnancy loss in mice was associated with perturbed IDO1-dependent maternal immune tolerance. https://doi.org/10.1289/EHP10640

Introduction

Humans are widely exposed to synthetic estrogen compounds, including bisphenol A (BPA). Oral ingestion of BPA, a component of epoxy resins and polycarbonate plastics found in food and drink packaging materials, is the primary route of human exposure.[1] Additional sources include inhalation from air and dust[2] as well as dermal exposure from thermal receipt papers.[3] Human BPA exposure level estimates range between 0.5 and 10 ng/mL in blood[4]; detectable levels of both free and total (i.e., conjugated plus unconjugated) BPA reported in urinary[5,6,7,8,9] and blood and serum[10,11,12] samples of pregnant women have been typically higher on average than those of nonpregnant adults. When ingested orally, the biologically active parent BPA compound undergoes rapid first-pass metabolism in the adult human liver predominantly to BPA glucuronide via uridine diphosphate glucuronosyltransferases. The inert BPA conjugate gets excreted primarily in urine within 12 h,[13] although detectable levels of unconjugated BPA found in urine,[14,15,16,17,18,19] circulation,[4,12,20] and within tissues of the maternal–fetal interface[12,21,22] suggest an inefficient deconjugation process that could leave humans at risk of adverse health effects.

Studies have shown that BPA exposure is positively correlated with higher rates of preterm birth,[23] preeclampsia,[24] and recurrent miscarriages in humans.[25,26] Pregnancy loss is of major concern given that 10–15% of clinically recognized pregnancies in the United States end in miscarriages,[27] and environmental exposures, particularly to endocrine disruptors, have been implicated in the etiology.[28] Reproductive toxicities linked to exposure to low doses of BPA include impaired quality, maturation, and production of mouse germ cells;[29,30] abnormal ovarian and uterine function in humans and animals; and perturbed embryonic and placental development in mice and human cell lines.[31,32]

Because of the concern for reproductive toxicity, BPA has been banned in the production of infant formula packaging, sippy cups, and baby bottles by the U.S. Food and Drug Administration,[33] the European Union,[34] and the Canadian government.[35] Many companies have switched to manufacturing BPA-free consumer products owing to public health concerns surrounding BPA toxicity. Although widespread efforts have been made to reduce BPA exposure from consumer products, structural BPA analogs that may share potential adverse reproductive effects are still largely used for commercial purposes. Tetrabromobisphenol A (TBBPA) is a brominated derivative of BPA found ubiquitously in the environment.[36] It is the highest production volume reactive and additive flame retardant worldwide used commonly in paper, textiles, furniture, and electronics and as a plasticizer in electronic coatings and adhesives.[37] Like BPA, oral ingestion is considered the main route for TBBPA exposure, although dermal contact and inhalation via contaminated household dust have been observed.[36] Human and rat studies demonstrate that TBBPA is readily metabolized and excreted as its glucuronidated or sulfate conjugated forms after oral administration and dermal absorption.[38] Accordingly, human exposure to TBBPA is estimated to be low[39] although on the rise owing to its increased production and use as evidenced by an increased estimated dietary TBBPA intake of 0.256[40] to 1.34 ng/kg BW per day[41] in 2007 and 2011, respectively. Detectable levels of TBBPA have been measured in human maternal serum, breast milk, and umbilical cord serum,[42] demonstrating maternal exposure and transplacental transfer of TBBPA to the developing fetus. To date, only one epidemiological study has been published that evaluates the link between TBBPA (1.260 ± 6.568 ng/mL in maternal serum) and pregnancy outcomes including low infant birth weight.[43] In animal studies, TBBPA exposure is associated with reduced reproductive success in zebrafish.[44]

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TBBPA exposure was also associated with lower embryonic weight and a higher number of fetal malformations and death upon oral administration in rats.[45] In a human first trimester placental cell line, TBBPA exposure altered the release of anti- and pro-inflammatory mediators and was associated with higher mRNA expression of inflammatory genes, all of which were factors important for pregnancy success.[46] Together, this evidence warrants further investigation of TBBPA for potential reproductive toxicity.

Despite human studies suggesting that BPA exposure is positively associated with a higher risk of pregnancy loss,[25,26] the mechanisms remain poorly understood. One cause of pregnancy loss is aberrant maternal–fetal immune tolerance.[47] Alterations in cellular,[48,49,50,51,52] hormonal,[53] molecular,[54,55,56,57,58,59] and genetic[60] mediators of immune tolerance have been linked to miscarriages. BPA[61,62] and TBBPA[46] exposure alters cellular and molecular components of the immune system in pregnancy. One proposed mechanism causatively linked to reduced maternal–fetal immune tolerance is disruptions in indoleamine 2,3-deoxygenase 1 (IDO1)–mediated tryptophan catabolism. IDO1 is the first and rate-limiting enzyme of tryptophan catabolism that converts tryptophan into kynurenine catabolites. Kynurenine drives expansion of regulatory T cells (Tregs),[63] which selectively inhibit proliferation and survival of effector T cells, including T helper 17 (Th17) cells, which would otherwise produce excessive pro-inflammatory responses against the semiallogeneic fetus.[64] Higher levels of Th17 cells have been reported in miscarriages[52] and unexplained recurrent pregnancy loss[65,66] in humans. Lower IDO1 protein and mRNA expression in humans[67] and enzymatic activity in mice[68] have been associated with pregnancy loss. Consistent with the role of IDO1 in Treg expansion, a lower percentage and number of Tregs are linked to pregnancy loss in humans[69,70,71,72] and mice.[73] In addition, lower levels of Tregs in peripheral blood[74] and the maternal spleen[75] have been associated with lower placental *Ido1* expression in mouse pregnancy loss. These observations imply that IDO1 activation and generation of kynurenine metabolites contribute to a favorable maternal–fetal immune environment that supports a successful pregnancy. Interestingly, elevated tryptophan levels have been reported in pregnant mice exposed to BPA;[76] however, no studies have characterized the potential link between altered tryptophan catabolism and pregnancy loss in the context of environmental exposure. The present study asked whether maternal exposure to BPA and TBBPA adversely influenced pregnancy success in mice through mechanisms related to perturbed Treg- and *Ido1*-associated maternal–fetal immune tolerance.

Materials and Methods

Dietary BPA and TBBPA Exposure

Six-to-10-wk-old virgin female mice were randomly assigned and exposed to one of the following diets manufactured by Envigo: *a*) control diet (TD 95092) made with 7% corn oil substituted for 7% soybean oil (modified AIN-93G diet), *b*) 50 mg/kg BPA diet (TD 09518), or *c*) 2.5 mg/kg TBBPA diet (TD 150780). The estimated daily exposure to BPA is 10 mg/kg body weight (BW) per day based on the average weight of female mice of 25 g and daily food consumption of 5 g. The exposure paradigm results in maternal serum BPA levels of 2 ng/mL.[77] The estimated daily dose of TBBPA, 500 μg/kg BW per day, is below its oral reference dose of 600 μg/kg BW per day for reproductive toxicity calculated based on uterine endometrial atypical hyperplasia observed in rats.[78,79] Female mice were exposed to the assigned diets for 2 wk prior to mating and time-mated in trios with unexposed males (i.e., 2:1 female to male ratio). The day in which a vaginal copulatory plug was observed was designated as E0.5. Exposure continued during mating and throughout pregnancy until E6.5–16.5, when the mice were euthanized using carbon dioxide asphyxiation, followed by opening of the chest cavity. Pregnancy rates for dams exposed to control, BPA, and TBBPA diets are presented in Table S1. The exposure paradigm is presented in Figure 1. A summary of data end points and sample sizes for each study can be found in Table 1. Mice were housed in XJ microisolator cages (Allentown) made with medical grade, chemically resistant Udel polysulfone (Solvay) and given *ad libitum* access to food and sterile water provided in glass bottles. The mouse housing facility remained at 74°F with a 12:12-h light:dark cycle (lights on at 0600 hours; lights off at 1800 hours). Mouse studies were performed in accordance with the institute for animal care and use committee at the University of Rochester Medical Center.

Generation of Allogeneic and Syngeneic Pregnancies

Both allogeneic and syngeneic pregnancies were used to study fetal resorption and immune cells. Allogeneic matings between strains of mice with distinct genetic backgrounds are more similar to human pregnancies. Paternally inherited genetic material in allogeneic pregnancies leads to fetal expression of proteins that are foreign to the mother, hence leading to tolerance mechanisms in the maternal immune system. Matings between genetically similar strains of mice represent immunocompatible syngeneic pregnancies. For the allogeneic pregnancy model, CBA/J (CBA) female mice were time-mated with C57BL/6 (B6) males, designated as CBAXB6 throughout this paper. B6 females time-mated to B6 males (B6XB6) were used as the syngeneic pregnancy model. CBA and B6 mice were purchased from JAX.

Fetal Hemorrhaging and Resorption Studies

Hemorrhaging in E7.5 conceptuses was positively linked to fetal resorption[68] and can therefore be used as an early indicator for fetal loss. Furthermore, as allogeneic pregnancies are more susceptible to fetal resorption relative to syngeneic pregnancies,[68,80,81,82,83,84] both pregnancy models were studied. Conceptuses from allogeneic and syngeneic pregnancies were harvested and analyzed microscopically at E7.5 for extensive hemorrhaging and scored by two individuals blinded as to the exposure group. Healthy-appearing conceptuses were scored as "nonhemorrhaging," whereas conceptuses that showed severe bloodiness and loss of normal structure as "hemorrhaging." At E16.5, fetuses from allogeneic pregnancies

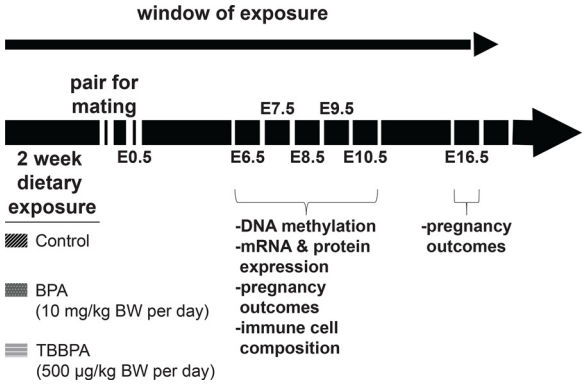

Figure 1. Schematic diagram of the exposure paradigm. Virgin female mice began exposure to control, 10 mg/kg BW per day bisphenol A (BPA), or 500 μg/kg BW per day tetrabromobisphenol A (TBBPA) diet for 2 wk prior to mating. Females were subsequently time-mated with unexposed males until a copulatory plug was detected and then separated from the male. Exposure continued during mating and throughout the pregnancy. Pregnant females were euthanized at the indicated gestational ages for tissue collection and analysis. Note: BW, body weight; E, embryonic day.

were scored for resorption. Rates of hemorrhaging and resorption were expressed as percentages of total conceptuses and fetuses, respectively.

Collection and Preparation of Immune Cells

Spleens were collected from nonpregnant adult female mice exposed to control, BPA, or TBBPA diets for 2 wk. For studies in pregnant mice, maternal spleens, and decidual capsules (the latter representing the maternal–fetal interface) were collected between E6.5 and 10.5. Decidual capsules were dissected under a light microscope and separated from the myometrium while keeping conceptuses intact. All decidual capsules from the same litter were pooled and processed into single-cell suspensions using a modified published protocol.[85] Briefly, tissues were minced in 0.02% collagenase D (Roche) in Hanks balanced salt solution (HBSS) and incubated at 37°C while rocking at 225 rpm in an orbital shaker. Spleens were minced in 2.5% fetal bovine serum in HBSS (FBS-HBSS). Red blood cells were lysed using an ammonium chloride solution. Afterward, all tissues were filtered through 70−μM filters into phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and 0.1% sodium azide (decidual capsules) or FBS-HBSS (spleens), washed, counted using a TC20 Automated Cell Counter (Bio-Rad), and resuspended at a maximum of 2×10^6 cells per 1 mL per flow cytometry tube in PBS.

Flow Cytometry

Cells isolated from spleen and decidual capsules were incubated with the LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen) for 30 min in the dark. Cells were subsequently incubated with purified rat antimouse CD16/CD32 (1:500; Cat. No. 553141; BD Biosciences) for 10 min to block nonspecific staining and subsequently stained for extracellular markers with optimal concentrations determined by titration of the following fluorochrome-conjugated monoclonal antibodies purchased from BD Biosciences: CD3 Molecular Complex [allophycocyanin (APC); clone 17A2; Cat. No. 565643], CD4 [fluorescein isothiocyanate (FITC); clone RM4-5; Cat. No. 553046], CD45.2 (APC-Cy7; clone 104; Cat. No. 560694), and CD25 (PE-CF594; clone PC61 Cat. No. 562694). After fixation and permeabilization using the Forkhead Box P3 (FOXP3)/Transcription Factor Staining Buffer Set (eBioscience), the cells were incubated with fluorochrome-conjugated antibodies against FOXP3 (AF700; clone FJK-16S; Cat. No. 56-5773-82; Invitrogen) and retinoid-related orphan receptor-gamma t (RORγt; PE; clone Q31-378; Cat. No. 562607; BD Biosciences) to identify Tregs and Th17

Table 1. Summary of data end points.

End points evaluated	Mating combination	Sample size	Figures
Allogeneic conceptus hemorrhaging	CBAXB6	Control: 7 dams, 36 conceptuses BPA: 6 dams, 32 conceptuses TBBPA: 4 dams, 21 conceptuses	2A-C
Syngeneic conceptus hemorrhaging	B6XB6	Control: 6 dams, 42 conceptuses BPA: 6 dams, 36 conceptuses TBBPA: 4 dams, 17 conceptuses	NA
Fetal resorption	CBAXB6	Control: 25 dams, 131 fetuses BPA: 20 dams, 90 fetuses TBBPA: 26 dams, 122 fetuses	2D
Allogeneic maternal spleen and decidual capsule Tregs, $CD4^+$ T cells, and Th17 cells	CBAXB6	Control: 21 dams BPA: 16 dams TBBPA:13 dams	3; S3; S4
Syngeneic maternal spleen and decidual capsule Tregs and $CD4^+$ T cells	B6XB6	Control: 18 dams BPA: 10 dams TBBPA: 12 dams	S5A–H
Syngeneic maternal spleen and decidual capsule Th17 cells	B6XB6	Control: 8 dams BPA: 6 dams TBBPA: 9 dams	S5I–L
Allogeneic and syngeneic Treg comparison in maternal spleen and decidual capsule	CBAXB6 and B6XB6	CBAXB6: 8 dams B6XB6: 9 dams	4
Nonpregnant CBA female Tregs and $CD4^+$ T cells	NA	Control: 8 dams BPA: 7 dams TBBPA: 8 dams	5
Ido1 placental mRNA expression	B6XPWD	Control: 10 dams, 8 M and 10 F placentas BPA: 11 dams, 11M and 10 F placentas TBBPA: 11 dams, 10 M and 11 F placentas	6A–C
Ido1 mRNA expression comparison among placenta, epididymis, and ileum	B6XPWD and B6XB6	placentas: 4 dams, 2 M and 2 F placentas ilea: 4 M cauda epididymides: 4 M	6D
IDO1 protein expression	B6XPWD	Control: 3 dams, 1 M and 2 F placentas, 453 cells BPA: 3 dams, 3 M placentas, 443 cells TBBPA: 3 dams, 3 F placentas, 490 cells	7D
Ido1 total DNA methylation	B6XPWD	Control: 10 dams, 9 M and 10 F placentas BPA: 11 dams, 11 M and 13 F placentas TBBPA: 11 dams, 10 M and 11 F placentas	8B–D
Ido1 maternal allele-specific DNA methylation	B6XPWD	Control: 3 dams, 3 F placentas[a] BPA: 3 dams, 3 M placentas TBBPA: 3 dams, 3 F placentas	8E,F

Note: Decidual capsules of the same exposure group and gestational age were pooled for Treg, $CD4^+$ T cell, and Th17 cell analysis, if needed, to increase cell number. BPA, Bisphenol A; B6, C57BL/6; CBA, CBA/J; F, female; *Ido1*, indoleamine 2,3 deoxygenase 1; M, male; NA, not applicable; PWD, PWD/PhJ; TBBPA, tetrabromobisphenol A; Th17, T helper 17; Treg, regulatory T cell.

[a]Mean percentage DNA methylation was similar between control male and female placentas (i.e., $48.9 \pm 19.8\%$ vs. $43.7 \pm 4.4\%$, respectively; $p = 0.8103$). Only female data are presented here.

cells, respectively, which differentiate from $CD4^+$ T lymphocytes.[86] Antibody dilutions are included in Table S3. An 18-color LSR-II cytometer (BD Biosciences) was used for data acquisition and FCS Express 7 flow cytometry software (De Novo Software) was used for data analysis. Fluorescence minus one controls were used to define gating parameters.

Sex Identification Polymerase Chain Reaction

To identify the fetal sex reflected in trophoblast cells of the mouse placenta, polymerase chain reaction (PCR) was performed in genomic (g) DNA extracted from yolk sac using a modified HotSHOT method.[87] Briefly, yolk sacs were incubated with a 25 mM sodium hydroxide solution containing 0.2 mM ethylenediaminetetraacetic acid for 1 h at 95°C. Afterward, an equal volume of a 40 mM Tris-HCl solution was added, and samples were centrifuged at 2,000 rpm for 10 min. Approximately 80 µL of supernatant were collected from each sample and used for PCR. X chromosome-specific gene *Kdm5c* and Y chromosome-specific gene *Kdm5d* were amplified in the gDNA samples using 10 µM structural maintenance of chromosomes-forward and -reverse (SMC-F and SMC-R) primers and GoTaq DNA Polymerase (Promega Corporation). The PCR conditions were as follows: 95°C for 5 min, 40 cycles at 95°C for 15 s, 55°C for 1 min, 72°C for 1 min, and, finally, 72°C for 7 min. The SMC primer sequences are as follows: forward primer 5′-TGA AGC TTT TGG CTT TGA G-3′ and reverse primer 5′-CCA CTG CCA AAT TCT TTG G-3′. Females were identified by a single 330-bp amplicon, whereas males were identified by two amplicons, 330 and 301 bp.

Generation of F1 Hybrid Mice for Molecular Analysis

For mRNA expression and DNA methylation studies, F1 hybrid progeny were generated by mating female B6 to male PWD/PhJ or PWD (JAX) mice, designated as B6XPWD throughout this paper. The B6 and PWD mice have single nucleotide polymorphisms (SNPs) in the exons and promoter of the *Ido1* gene that enable molecular analysis distinguishing the maternal and paternal alleles.[88] Placentas were harvested from E9.5 B6XPWD F1 hybrid mice, separated carefully from the yolk sac, and isolated from decidua to minimize maternal contamination. Tissues were frozen in a dry ice and methanol bath and stored at −80°C until analysis. Placentas from each litter were sexed prior to analysis, and 1–2 male and female placentas per litter were randomly selected for the studies.

mRNA Expression Studies

Total RNA was extracted from E9.5 B6XPWD F1 placentas and caput epididymides and ilea from unexposed adult B6 male mice using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's protocol. Extracted RNA was quantified using the NanoDrop One spectrophotometer and evaluated for purity using the A260/A280 ratio with an acceptable range of 2.0–2.2. Superscript IV reverse transcriptase, deoxynucleotide triphosphates, and random hexamers (Invitrogen) were used to generate complementary deoxyribonucleic acid (cDNA) from 1,000 ng RNA via a reverse transcriptase polymerase chain reaction. A negative control reaction with no Superscript IV enzyme was included to assess DNA contamination of RNA. Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted to measure total gene expression in E9.5 placentas, caput epididymides, and ilea using a QuantStudio 5 RT-PCR System (Applied Biosystems). The RT-qPCR protocol is as follows: 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems), 0.16 µL of 25 µM reverse and forward primers, 7.68 µL of nuclease-free water, and 2 µL of cDNA (40 ng), with an annealing temperature of 60°C. The following *Ido1* primers were used: forward primer 5′-AGTCGGAAGAGCCCTCAAAT-3′ and reverse primer 5′-TGCCAGCCTCGTGTTTTATT-3′ (176 bp amplicon). All samples were measured in duplicates and analyzed using QuantStudio Design & Analysis Software (Applied Biosystems). The Δ cycle threshold (Ct) for *Ido1* was calculated by normalizing averaged Ct values to the housekeeping gene RNA polymerase II subunit A or *Polr2a*.[89] Relative quantities were obtained using the $2^{-\Delta CT}$ comparative CT method[90] and presented on a $\log_2$ scale. The reference gene primer sequences are as follows: forward primer 5′-TGCAAGAGGAGGAAGAGGTG-3′ and reverse primer 5′-AGCATGTTGGACTCAATGCA-3′ (73 bp amplicon). *Ido1* mRNA expression was analyzed in male and female placentas separately and combined.

Immunofluorescence Staining

Double immunofluorescence staining was used to measure differences in fluorescence intensity of IDO1 that was restricted to parietal trophoblast giant cells in E9.5 placentas.[91] To confirm the cell-specific localization of IDO1, an antibody for placental lactogen I (PL1), a protein highly and specifically expressed in parietal trophoblast giant cells in E9.5 placenta,[92,93,94] was included in the study. Isolated decidual capsules with myometrium intact were fixed in 10% neutral buffered formalin (Sigma-Aldrich) at 4°C for 24 h, paraffin-embedded, bivalved in sagittal section through the mid placental plane, and serially sectioned (5 µM thick) for mounting. Slides were dewaxed in xylene and rehydrated through graded ethanol to distilled water. Slides were immersed in sodium citrate buffer (pH 6.0) in a 96°C water bath for 15 min and membrane permeabilized for 10 min with 0.2% Triton X-100 in PBS. To quench autofluorescence, slides were incubated with 0.1 M glycine for 90 min, followed by blocking with 5% normal goat serum in PBS with 1% BSA for 1 h at room temperature. Sequential rounds of primary (overnight at 4°C) and fluorophore-conjugated secondary (2 h at room temperature) antibody incubations were performed. Primary antibodies used were mouse anti-IDO H-11 (1:100; sc-137,012; Santa Cruz Biotechnology) and mouse antiplacental lactogen I C-12 (1:50; sc-376,436; Santa Cruz Biotechnology). Cross-adsorbed secondary antibodies used were 1:500 goat antimouse immunoglobulin (Ig) G1 Alexa Fluor 488 (Invitrogen) and 1:500 goat antimouse IgM heavy chain Alexa Fluor 594 (Invitrogen). Slides were washed with Millipore water for 3×5 min after each individual antibody incubation. All antibodies were diluted in PBS with 1% BSA and 0.1% Tween-20. Nuclear counterstaining was performed using 300 nM 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Relative levels of IDO1 and PL1 staining intensities were quantified using the corrected total cell fluorescence (CTCF) method,[95] with measurements taken using ImageJ (version 1.48).[96] Briefly, a region of interest was created by tracing the trophoblast giant cell using the PL1 channel of the multichannel image. For IDO1 measurements, the region of interest was superimposed onto the IDO1 channel; integrated density and area of trophoblast giant cells as well as background mean gray values of three equal-area circles in the decidua were measured. The following formula was used to calculate IDO1 and PL1 immunostaining intensities: CTCF = integrated density of traced trophoblast giant cell − (area of trophoblast giant cell × average of the mean gray value of background readings). The CTCF of all trophoblast giant cells were averaged within each exposure group. A visual representation of the IDO1 and PL1 intensity analysis and quantification of PL1 can be found in Figure S1A–D and S1E, respectively. Representative images of IDO1 and PL1 staining in decidual capsules from control and BPA- and TBBPA-exposed mice are shown in Figure S2A–C.

Ido1 Knockout Studies

Ido1$^{-/-}$ mice (B6.129 – Ido1^{tm1Alm}/J; Stock No. 005867) were purchased from JAX and maintained in the vivarium by setting up heterozygotes into breeding pairs. To generate homozygous F1 progeny for IDO1 immunofluorescence staining, *Ido1*$^{-/-}$ females were time-mated with *Ido1*$^{-/-}$ males, and decidual capsules, which contain the decidua and whole conceptus, were collected from pregnant females at E9.5. Mice were genotyped using the KAPA2G Fast HotStart PCR Kit (KAPA Biosystems) following protocol 35022 (JAX). The PCR conditions were as follows: 94°C for 3 min, 10 cycles of 94°C for 15 s, 65°C for 15 s (−0.5°C/cycle), 68°C for 1 s, 28 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 1 s. The protocol ends with a final extension step at 72°C for 30 s. The primer sequences are as follows: mutant forward primer 5′-CGTGCAATCCATCTTGTTCA-3′, wild-type (WT) forward primer 5′-TATTGAAAGGGGAATCCAGA-3′, and common reverse primer 5′-GTGTCAGAAAGCTCACTGCTT-3′. WT and mutant alleles were identified as having a 252- or 575-bp amplicon, respectively, whereas heterozygotes have both amplicons.

DNA Methylation Studies

gDNA was extracted from E9.5 B6XPWD F1 placentas using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's protocol, quantified using the NanoDrop One spectrophotometer, and subsequently bisulfite treated using the EpiTect Fast DNA Bisulfite kit (Qiagen). DNA quality was assessed using the A260/A280 ratio with the acceptable range of 1.8–2.0. *Ido1* is a maternally expressed imprinted gene in the placenta; its allelic expression pattern is linked to a paternally methylated promoter differentially methylated region (DMR).[88] Because BPA exposure influenced DNA methylation of imprinted genes,[77,97] total DNA methylation analysis of *Ido1* was performed using pyrosequencing, a high-throughput and quantitative sequencing-by-synthesis system, with the PyroMark Advanced Q24 (Qiagen). The published *Ido1* m08 and m17 assays, which included 6 CpG sites within the *Ido1* DMR, were used.[88] These sites were denoted CpG sites 2–7 in the present paper to be consistent with the method published by Spinelli et al. in 2019.[88] Male and female placentas were analyzed for total DNA methylation levels separately and combined. Allele-specific DNA methylation analysis of CpG sites 2, 3, 4, 5, and 7 was performed using bisulfite clonal sequencing with a 55°C annealing temperature that preferentially amplifies the maternal *Ido1* DMR allele.[88] The paternal *Ido1* DMR allele was not analyzed. CpG site 6 could not be distinguished as unmethylated (TG) vs. a strand of PWD origin (TG) owing to the presence of a C/T SNP between the B6 and PWD mice.

Statistical Analysis

Data were statistically analyzed to compare differences in means across exposure groups using Prism (version 8; Graphpad). One-way analyses of variance, followed by Dunnett's multiple comparisons post hoc tests, were used. Data that violated assumptions of normality were analyzed using the Kruskal-Wallis and Dunn's tests. E7.5 hemorrhaging and E16.5 fetal resorption data were analyzed using the chi-square test for all exposure groups and Fisher's exact tests to compare BPA and TBBPA groups to the control, separately. Two-tailed unpaired *t*-tests were conducted to analyze differences in mean percentages and numbers of Tregs and CD4$^+$ T cells between allogeneic and syngeneic pregnancies. $p \le 0.05$ were considered statistically significant throughout the study. Data are presented as mean ± standard error of the mean (SEM) unless noted otherwise.

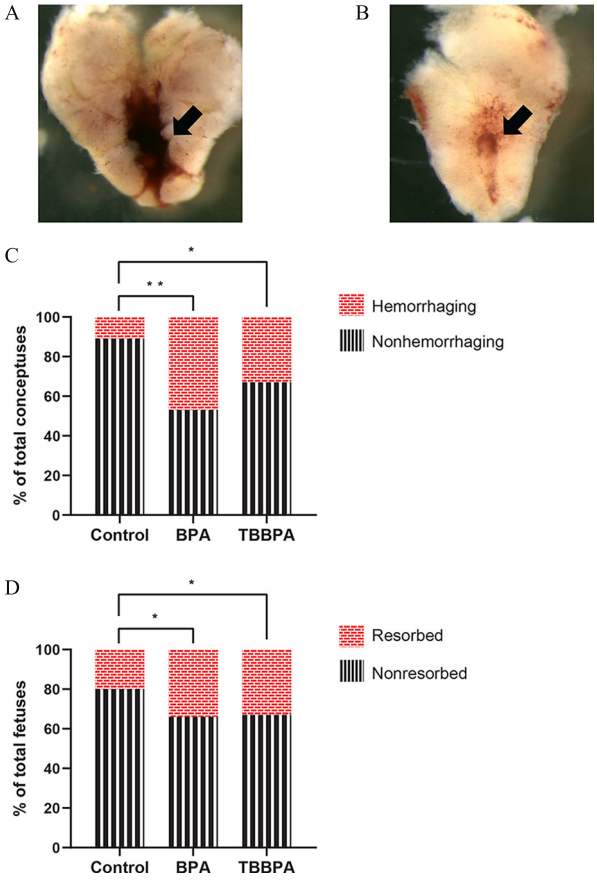

Figure 2. Effects of BPA and TBBPA exposure on pregnancy outcomes. Representative microscopic images of (A) hemorrhaging and (B) nonhemorrhaging embryonic day (E) 7.5 conceptuses within the dissected decidua tissue. Arrows indicate the location of the conceptus. The nonhemorrhaging conceptus appeared normal, in contrast to the extensive bleeding surrounding the hemorrhaging conceptus. (C) Effect of exposure on rates of conceptus hemorrhaging. The *y*-axis represents the percentage of total conceptuses with hemorrhaging (red, brick) or no hemorrhaging (black, stripes). Control: 11.1% (4 of 36), BPA: 46.9% (15 of 32), TBBPA: 33.3% (7 of 21) hemorrhaging conceptuses. The number of conceptuses (*n*) analyzed was 21–36, representing 4–7 dams (*N*). (D) Effect of exposure on fetal resorption rates at E16.5. Control: 19.8% (26 of 131), BPA: 34.4% (31 of 90), TBBPA: 32.8% (40 of 122) resorbed fetuses. The number of fetuses (*n*) analyzed was 90–131, representing 20–26 dams (*N*). The *y*-axis represents the percentage of total resorbed (red, brick) or nonresorbed (black, stripes) fetuses. All data were analyzed using the chi-square test for all exposure groups and Fisher's exact tests to compare BPA and TBBPA groups to the control, separately. **, $p \le 0.01$; *, $p \le 0.05$. Note: BPA, bisphenol A; TBBPA, tetrabromobisphenol A.

Results

Assessment of Pregnancy Loss

To determine whether BPA and TBBPA exposure was associated with higher rates of pregnancy loss in mice, allogeneic CBAXB6 conceptuses were harvested at E7.5, and the proportion of hemorrhaging conceptuses (Figure 2A) vs. the nonhemorrhaging ones that appeared healthy with no obvious blood loss (Figure 2B) was compared. In the control group, 11.1% (4 of 36) of allogeneic conceptuses had severe bleeding and were scored as hemorrhaging (Figure 2C). In the BPA and TBBPA groups, the rates of hemorrhaging were significantly higher than controls, that is, 46.9% (15 of 32) and 33.3% (7 of 21), respectively (Figure 2C).

To determine whether syngeneic pregnancies were impacted differentially, hemorrhaging rates were assessed in control and BPA- and TBBPA-exposed B6 females mated to B6 males. Analysis of E7.5 syngeneic conceptuses revealed no evidence of

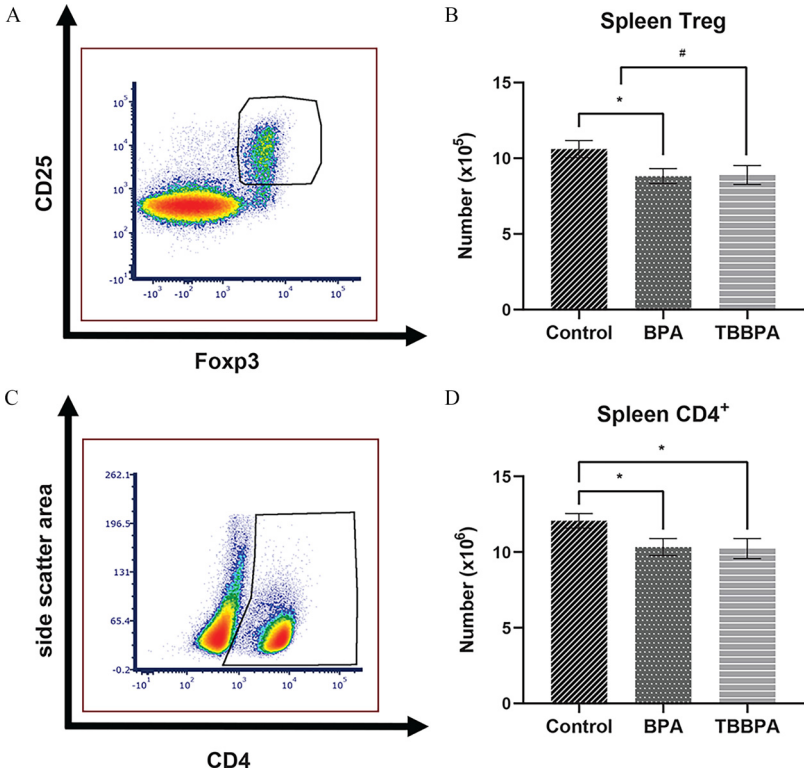

Figure 3. Effects of BPA and TBBPA exposure on Tregs and $CD4^+$ T cells in maternal spleen from allogeneic pregnancies. Spleen cells from control (black, diagonal stripes), BPA (dark gray, dots), and TBBPA-exposed (light gray, horizontal stripes) CBA/J (CBA) female mice mated to C57BL/6 (B6) males (i.e., CBAXB6) were harvested between E7.5 and 10.5 and processed for flow cytometry. Representative plots gated for (A) $CD25^+FOXP3^+$ Tregs and (C) $CD4^+$ T cells. The graphs show mean number ± SEM of (B) Tregs and (D) $CD4^+$ T cells. Sample sizes range from 13–21 mice per group. Data were analyzed using ANOVA, followed by Dunnett's multiple comparison test. See numeric data in Table S4. *, $p \le 0.05$; #, $p = 0.07$. Note: ANOVA, analysis of variance; BPA, bisphenol A; E, embryonic day; SEM, standard error of the mean; TBBPA, tetrabromobisphenol A; Treg, regulatory T cell.

hemorrhaging in control conceptuses, nor was there any difference among the BPA and TBBPA groups, that is, rates for each exposure group were 0.0% (Control: 0 of 43, BPA: 0 of 48, TBBPA: 0 of 28). These findings demonstrated that BPA and TBBPA exposure in allogeneic pregnancies, but not syngeneic pregnancies, was associated with higher rates of conceptus hemorrhaging.

To study whether higher rates of hemorrhaging in conceptuses from BPA- and TBBPA-exposed allogeneic pregnancies were associated with higher rates of fetal loss, resorption rates were assessed at E16.5. The rates of resorbed fetuses were significantly higher in the BPA and TBBPA groups relative to controls, that is, 34.4% (31 of 90), 32.8% (40 of 122), and 19.8% (26 of 131), respectively ($p = 0.0235$; Figure 2D), demonstrating that E7.5 hemorrhaging was positively correlated to E16.5 fetal loss in BPA- and TBBPA-exposed allogeneic pregnancies.

Evaluation of Tregs during Pregnancy

To determine whether higher rates of fetal resorption in BPA- and TBBPA-exposed allogeneic pregnancies was associated with lower levels of Tregs, the percentage and number of Tregs in maternal spleens and decidual capsules were measured using flow cytometry. Tregs were defined as $CD45^+CD3^+CD4^+CD25^+FOXP3^+$ cells (Figure 3A). Representative flow cytometry plots show gating for Tregs in maternal spleens (Figure S3A) and decidual capsules (Figure S4A). In allogeneic pregnancies, maternal spleens from BPA-exposed dams had a significantly lower mean Treg number (Figure 3B) but not percentage (Figure S3B) relative to controls. A trend in lower Treg number was noted in maternal spleens from TBBPA-exposed dams (Figure 3B). BPA and TBBPA exposure did not affect Treg percentage (Figure S4B) or number (Figure S4C) in decidual capsule cells. The study showed that exposure-induced higher rates of hemorrhaging in allogeneic conceptuses was linked to a lower number of maternal Tregs. Studies in syngeneic pregnancies showed no significant effects of BPA and TBBPA exposure on Treg percentage or number in maternal spleens (Figure S5A,B) and decidual capsules (Figure S5C,D), consistent with the absence of conceptus hemorrhaging.

Baseline differences in hemorrhaging rates between control-exposed allogeneic and syngeneic pregnancies (i.e., 11.1% or 4 of 36 vs. 0.0% or 0 of 43, respectively) were interesting, and a study was performed to independently assess whether the different mating combinations showed inherent differences in percentages and numbers of Tregs. Mean Treg percentage (Figure 4A) and number (Figure 4B) in maternal spleens from control-exposed allogeneic pregnancies were significantly lower relative to syngeneic (7.4% vs. 11.9% and 8.4×10^5 vs. 1.2×10^6, respectively). In decidual capsules, a lower percentage (Figure 4C) of Tregs was observed but not a lower number (Figure 4D). The observation demonstrated that the proportion of maternal Tregs was inversely correlated with rates of conceptus hemorrhaging such that lower Tregs in allogeneic control pregnancies correlated with higher baseline rates of hemorrhaging.

Evaluation of $CD4^+$ T Cells and Th17 Cells

To determine whether differences in Tregs from BPA and TBBPA exposure reflected baseline differences in the $CD4^+$ T lymphocyte population, the percentage and number of $CD4^+$ T cells were measured. Representative flow cytometry plots show gating for $CD4^+$ T cells (Figures 3C, S3C, and S4D). Maternal spleens from BPA- and TBBPA-exposed allogeneic pregnancies

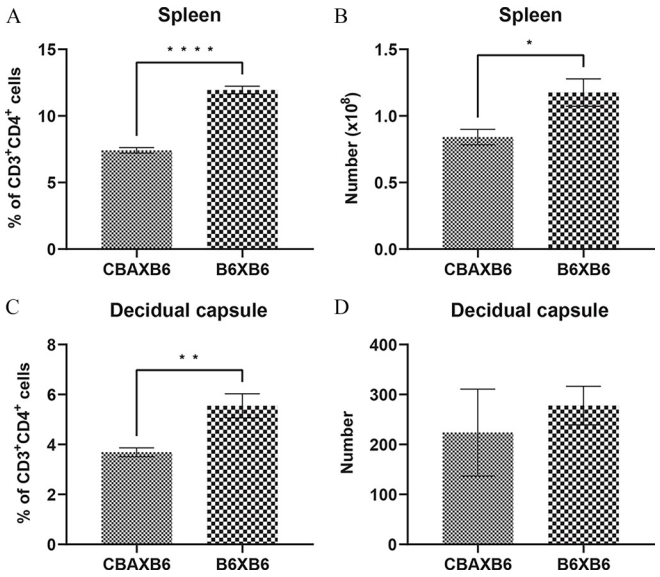

Figure 4. Comparison of regulatory T cells (Tregs) in maternal spleens and decidual capsules from allogeneic (CBAXB6) and syngeneic (B6XB6) pregnancies. Cells from E6.5–9.5 maternal spleens and E7.5–9.5 decidual capsules were processed for flow cytometry. Mean percentage and number (±SEM) of Tregs in (A,B) maternal spleens and (C,D) decidual capsules from allogeneic (CBAXB6; small, checkered pattern) and syngeneic (B6XB6; large, checkered pattern) pregnancies. Spleens and decidual capsules from 7–9 dams (*N*) were analyzed per group. Data were analyzed using an unpaired two-tailed *t*-test. See numeric data in Table S4. *, $p \le 0.0001$; **, $p \le 0.01$; *, $p \le 0.05$. Note: E, embryonic day; SEM, standard error of the mean.

had a significantly lower mean number of $CD4^+$ T cells (Figure 3D) but not a lower percentage (Figure S3D) relative to controls, suggesting that the lower Treg number in spleens from BPA- and TBBPA-exposed mice (Figure 3B) was associated with a lower number of $CD4^+$ T cells. No differences within decidual capsule $CD4^+$ T cells were observed (Figure S4E,F). No effects of BPA and TBBPA exposure on percentage and number of $CD4^+$ T cells in maternal spleens (Figure S5E,F) and decidual capsules (Figure S5G,H) in syngeneic pregnancies were observed.

To determine whether BPA and TBBPA exposure in allogeneic pregnancies was associated with higher abundance of Th17 cells, the percentage and number of Th17 cells in maternal spleens and decidual capsules were measured. Th17 cells were identified as $CD3^+CD4^+ROR\gamma t^+$; representative flow cytometry plots are shown in Figures S3A and S4A. No differences in the mean percentage or number of Th17 cells were observed among exposure groups in maternal spleens (Figure S3E,F) or decidual capsules (Figure S4G,H) from allogeneic pregnancies. A similar observation was noted in syngeneic pregnancies (Figure S5I–L).

Evaluation of Tregs in Nonpregnant Mice

The exposure window in this study began 2 wk prior to mating; therefore, the effects of BPA and TBBPA exposure on Tregs and $CD4^+$ T cells potentially reflected changes that occurred prior to pregnancy. To determine whether lower number of Tregs and $CD4^+$ T cells in maternal spleens from BPA and TBBPA exposure groups was pregnancy-specific, flow cytometry was performed using spleen cells harvested from nonpregnant CBA female mice. No differences in the percentages and numbers of Tregs (Figure 5A,B) and $CD4^+$ T cells (Figure 5C,D) were detected in spleens from control and BPA- and TBBPA-exposed CBA mice, demonstrating that lower Treg and $CD4^+$ T cell number was specific to pregnancy.

Analysis of Placental Ido1 Expression

To determine whether lower Treg number in BPA- and TBBPA-exposed mice was associated with lower *Ido1* mRNA expression, we performed RT-qPCR in E9.5 whole placentas from control

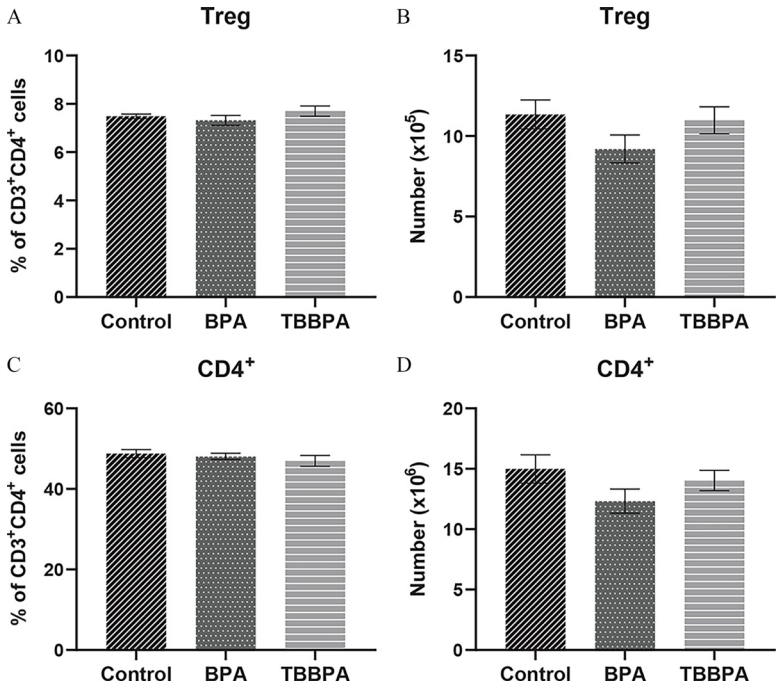

Figure 5. Effects of BPA and TBBPA exposure on Tregs and $CD4^+$ T cells in nonpregnant CBA female mice. Cells from spleens harvested from control (black, diagonal stripes), BPA (dark gray, dots), and TBBPA-exposed (light gray, horizontal stripes) nonpregnant CBA female mice were processed for flow cytometry. Mean percentage and number (±SEM) of (A,B) Tregs and (C,D) $CD4^+$ T cells in spleens. Spleens from 7–8 dams (*N*) were analyzed per group. Data were analyzed using ANOVA, followed by Dunnett's multiple comparison test. See numeric data in Table S4. Note: ANOVA, analysis of variance; BPA, bisphenol A; SEM, standard error of the mean; TBBPA, tetrabromobisphenol A; Treg, regulatory T cell.

and BPA- and TBBPA-exposed dams. For this study, and all DNA methylation analysis described below, we used F1 hybrid B6XPWD placentas (see the "Materials and Methods" section). No significant differences were observed in *Ido1* mRNA among exposure groups (Figure 6A–C). The relative expression of *Ido1* mRNA in whole placenta represents the gene's average expression among all cells in the tissue. Compared with tissues that express *Ido1* highly (e.g., the epididymis and ileum), mRNA levels in whole E9.5 placentas were lower (Figure 6D). Differences in *Ido1* mRNA among control, BPA, and TBBPA groups could have been masked owing to the cellular complexity of the E9.5 placenta. To measure IDO1 protein expression in trophoblast giant cells, dual immunofluorescence staining was performed in E9.5 placentas. To test the specificity of the IDO1 antibody, mice with the engineered deletion of the *Ido1* gene ($Ido1^{-/-}$ were included in the study. IDO1 staining was detected in trophoblast giant cells from WT (Figure 7A) but not $Ido1^{-/-}$ mice (Figure 7B), demonstrating the specificity of the antibody. Dual immunofluorescence revealed that IDO1 co-localized with PL1 in parietal trophoblast giant cells (Figure 7A). More importantly, IDO1 immunostaining intensities in trophoblast giant cells in E9.5 placentas from BPA- and TBBPA-exposed mice were lower than from controls (Figure 7C,D). The results showed that a lower Treg number in BPA- and TBBPA-exposed mice was associated with a lower IDO1 expression in trophoblast giant cells.

Ido1 Methylation Analysis in Placentas

We tested whether lower IDO1 expression in BPA- and TBBPA-exposed placentas was linked to altered *Ido1* DNA methylation in E9.5 placentas. Pyrosequencing analysis of the *Ido1* DMR (Figure 8A) revealed that placentas from BPA-exposed mice had a higher percentage of total DNA methylation at CpG sites 4 and 7 relative to controls (Figure 8B); however, no effects were detected from TBBPA exposure (Figure 8B). To determine whether there were sex-specific epigenetic effects of BPA and TBBPA exposure, male and female placentas were analyzed separately. In the BPA group, male placentas had higher percentage *Ido1* DNA methylation relative to controls at CpG sites 4, 5, 6, and 7 (Figure 8C). BPA exposure did not influence DNA methylation in females (Figure 8D). In the TBBPA group, no significant effects were observed in the male placentas (Figure 8C), but the female placentas had a significantly higher percentage of DNA methylation at CpG site 4 relative to controls (Figure 8D).

To test the possibility that a higher percentage of total *Ido1* DNA methylation in placentas from BPA- and TBBPA-exposed dams was associated with a gain of methylation at the maternal DMR, DNA methylation at the maternal *Ido1* DMR was measured using bisulfite clonal sequencing in E9.5 B6XPWD F1 placentas that carry SNPs enabling allele-specific analysis. BPA and TBBPA exposure was associated with higher methylation levels of the maternal *Ido1* DMR relative to controls (Figure 8E,F). Mean percentage DNA methylation levels in placentas from control, BPA, and TBBPA groups were $43.7 \pm 4.4\%$, $80.4 \pm 2.0\%$, and $88.9 \pm 4.5\%$, respectively ($p = 0.0003$). Overall, these studies showed that a lower IDO1 expression in trophoblast giant cells from BPA- and TBBPA-exposed mice was associated with total and maternal allele-specific hypermethylation of the *Ido1* DMR in the placenta.

Discussion

The present study provides evidence of reproductive toxicity from maternal exposure to BPA and TBBPA in mice. The administered

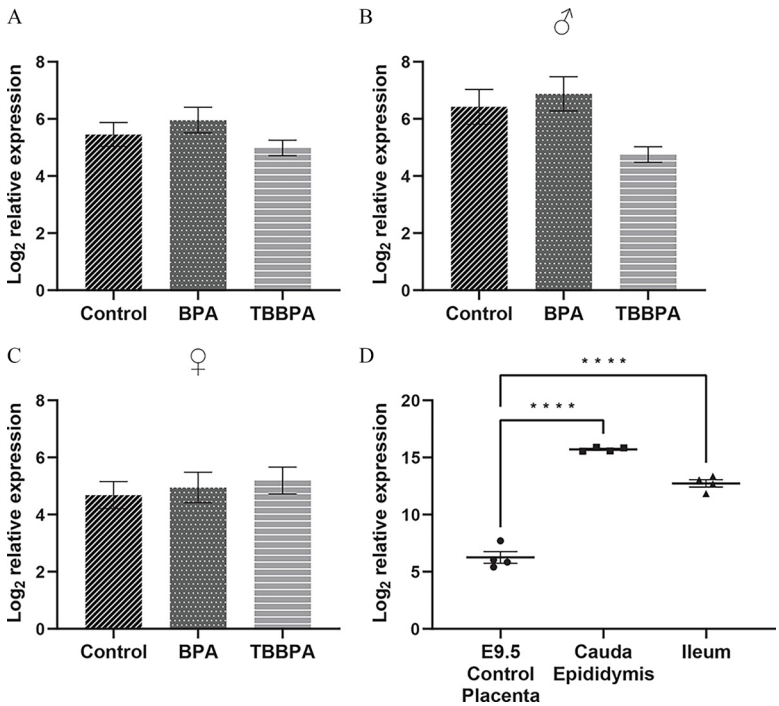

Figure 6. RT-qPCR analysis of indoleamine 2,3 deoxygenase 1 (*Ido1*) mRNA expression. (A–C) E9.5 placentas from B6 female mice exposed to control (black, diagonal stripes), BPA (dark gray, dots), and TBBPA (light gray, horizontal stripes) and mated to PWD/PhJ (PWD) males (i.e., B6XPWD) were analyzed for expression of *Ido1* relative to RNA polymerase II subunit A (*Polr2a*). E9.5 (A) combined male and female, (B) male, and (C) female placentas are shown. Data are displayed as mean expression ± SEM on the *y*-axis. Sample sizes range from 8 to 11 male and 10 to 11 female placentas. (D) Comparison of *Ido1* expression in E9.5 B6XPWD F1 placentas from control-exposed dams and epididymides and ilea from unexposed adult B6 male mice. $N = 4$ samples per group. Data presented on a $\log_2$ scale were normalized to the housekeeping gene *Polr2a*, and relative expression was calculated using the $2^{-\Delta CT}$ method. Data were analyzed using ANOVA or Kruskal-Wallis test, followed by Dunnett's or Dunn's multiple comparison test, respectively, when appropriate. See numeric data in Table S5. ****, $p \leq 0.0001$. Note: ANOVA, analysis of variance; BPA, bisphenol A; E, embryonic day; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of the mean; TBBPA, tetrabromobisphenol A.

dose of BPA (10 mg/kg BW per day) in the study was above the oral reference dose established by the U.S. EPA (i.e., 50 μg/kg BW per day);[98] however, levels of unconjugated BPA in serum from mice exposed to the paradigm (2.0 ± 0.4 ng/mL)[77] were within the physiological range measured in nonpregnant adults and pregnant women (i.e., 0.5–10 ng/mL).[4] The bioactive unconjugated form of BPA has been repeatedly detected in human blood,[4,12,20] urine,[14,15,16,17,18,19] breast milk,[99,100,101,102] amniotic fluid,[20,21,103] and follicular fluid,[21,104] as well as in the placenta[12] and fetal blood,[12,21,22] in some cases at levels higher than in the mother.[21,22] In addition, recent studies suggested that human exposure levels may have been grossly underestimated owing to indirect analytical techniques involving highly inefficient deconjugation of the chemical[105,106] and new urinary BPA estimates in pregnant women reported to be 44 times higher than previous levels reported for adults in the *Ido1*$^{-/-}$ mice.[107] These findings suggest that BPA can escape the rapid first-pass metabolism that regulatory agencies have used as evidence of low potential for toxicity owing to minimal bioavailability. Further evidence shows that BPA can be reactivated at the maternal–fetal interface given that human placentas had high β–glucuronidase activity, an enzyme responsible for BPA deconjugation.[108] The relevance of rodent models for BPA toxicity studies has also been called into question owing to inherent differences in BPA metabolism between rodents and humans (i.e., clearance primarily via bile/feces vs. urine, respectively). A 2011 study using rhesus monkeys as a surrogate for humans, however, showed that BPA pharmacokinetics was similar between mice and humans.[109] Efforts have been made by the Consortium Linking Academic and Regulatory Insights on Toxicity of BPA (CLARITY-BPA) to resolve discrepancies in reported adverse effects associated with BPA exposure;[106] adverse reproductive effects have been reported at doses as low as 2.5 μg/kg BW per day in rats.[110] Additional studies have provided evidence of low-dose BPA reproductive hazards in human and animal tissues and cell lines.[32,111] Overall, reevaluation of the assumption that

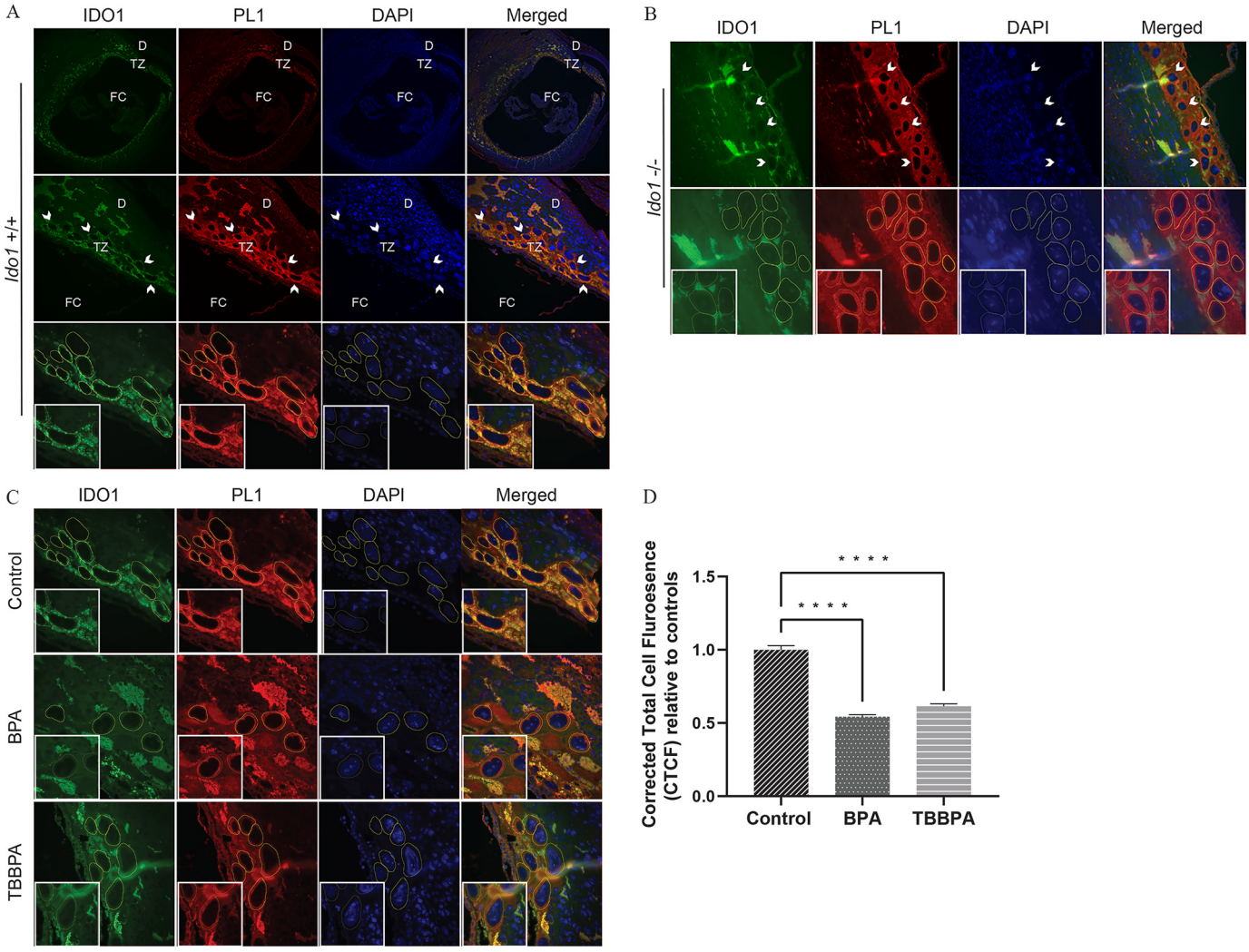

Figure 7. Immunofluorescence analysis of IDO1 protein expression in trophoblast giant cells. (A) Representative ×4 (top row), ×20 (middle row), and ×40 (bottom row) images of E9.5 B6XPWD F1 decidual capsules from *Ido1*$^{+/+}$ mice stained for IDO1, placental lactogen I (PL1) and DAPI. (B) Representative ×20 (top row) and ×40 (bottom row) images of an E9.5 *Ido1*$^{-/-}$ decidual capsule stained for IDO1, PL1, and DAPI. Arrowheads and light yellow outlines indicate representative trophoblast giant cells. (C) Representative ×40 images of stained E9.5 B6XPWD F1 decidual capsules from control and BPA- and TBBPA-exposure groups. Trophoblast giant cells outlined in light yellow were analyzed. (D) The CTCF method was used to calculate trophoblast giant cell IDO1 fluorescence intensity in the E9.5 decidual capsules. Mean CTCF ± SEM of IDO1 relative to controls is represented on the *y*-axis. (Control: 1.0 ± 0.0, BPA: 0.5 ± 0.0, TBBPA: 0.6 ± 0.0; Control (black, diagonal stripes), BPA (dark gray, dots), and TBBPA (light gray, horizontal stripes) groups are each represented by three dams, and 443–490 cells were analyzed per exposure group. Data were analyzed using Kruskal-Wallis test, followed by Dunn's multiple comparison test. ****, $p \leq 0.0001$. Note: BPA, bisphenol A; D, decidua; E, embryonic day; CTCF, corrected total cell fluorescence; DAPI, 4′,6-diamidino-2-phenylindole; FC, fetal compartment; SEM, standard error of the mean; TBBPA, tetrabromobisphenol A; TZ, trophoblast giant cell zone.

human BPA exposure is negligible and further characterization of low-dose effects of BPA on pregnancy outcomes in clinical and animal models are warranted.

Increasing concerns over BPA as a hazard to human health and efforts to reduce its usage in consumer products warrant investigation of potential toxicities from BPA analogs, including TBBPA, whose usage have been on the rise.[36,112] Studies have suggested that TBBPA exposure levels to the general population are low;[113] however, the elimination half-life of TBBPA is significantly longer (i.e., 2–3 d) than that of BPA.[114] Although studies on human TBBPA exposure levels are fewer relative to those on BPA, TBBPA was detectable in maternal and umbilical cord serum[42] and breast milk,[40,115] suggesting significant exposures for pregnant women and developing offspring. No epidemiological or clinical data currently exists linking TBBPA to pregnancy loss. In rats,[116] mice,[117] and frogs,[118] potential for toxicity to the male reproductive system have been reported. No marked adverse effects on pre- and postimplantation loss of pups in pregnant Sprague-Dawley rats were observed at oral doses between 100 and 1,000 mg/kg BW per day.[119] Exposure to

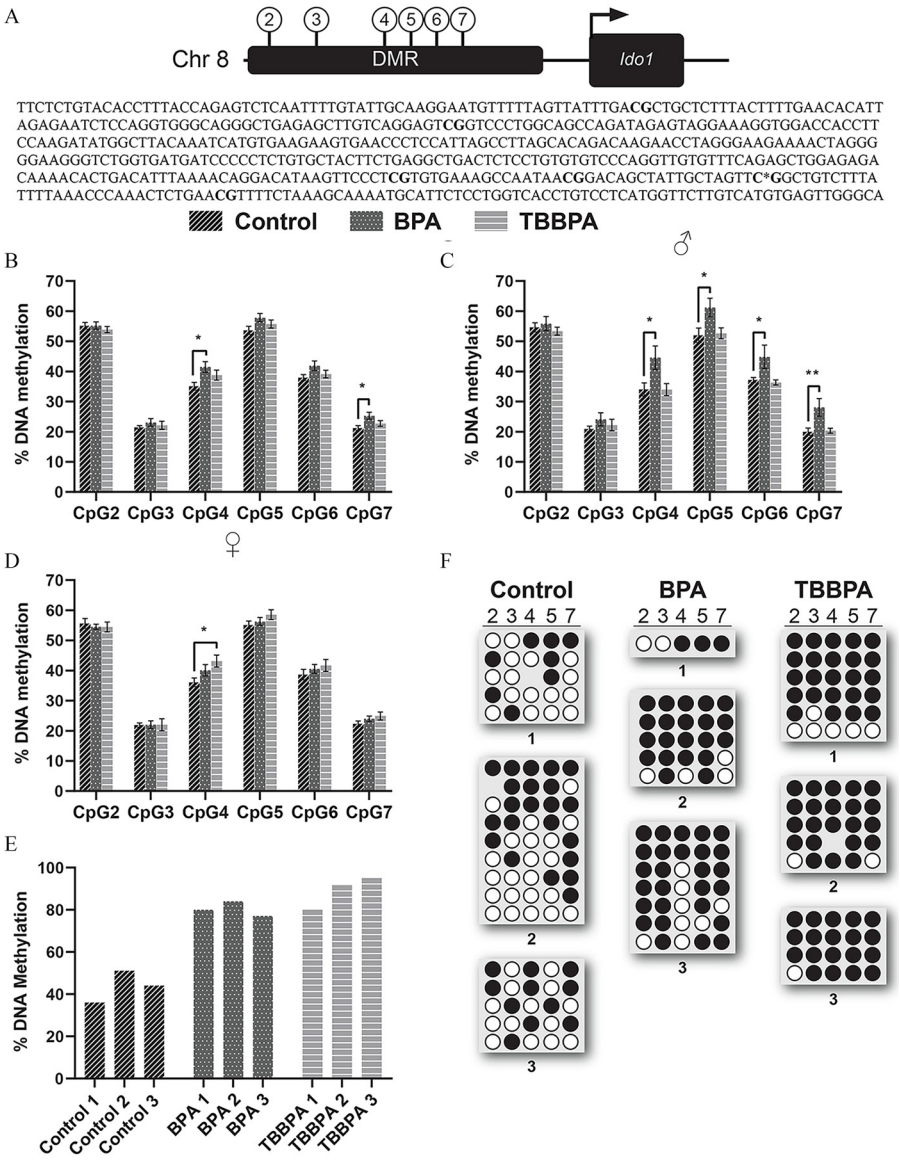

Figure 8. DNA methylation analysis at the *Ido1* DMR in placentas from control and BPA- and TBBPA-exposed mice. (A) Six CpG sites located within the *Ido1* DMR were assayed by pyrosequencing and bisulfite clonal sequencing. CpG site 6 was not analyzable in the bisulfite clonal sequencing because it contained a C/T SNP (indicated by an asterisk in the sequence). (B–D) Pyrosequencing results at CpG sites 2–7 in placentas from control (black, diagonal stripes), BPA (dark gray, dots), and TBBPA-exposed (light gray, horizontal stripes) dams. E9.5 (B) combined male and female, (C) male and (D) female placentas are shown. Mean percentages of DNA methylation ± SEM on the *y*-axis are shown. Sample sizes range from 8 to 13 placentas per exposure group for sex-specific analysis. Data were analyzed using ANOVA or Kruskal-Wallis test, followed by Dunnett's or Dunn's multiple comparison test, respectively, when appropriate. See numeric data in Table S6. *, $p \le 0.05$ for all statistical analysis. (E,F) Methylation levels of the maternal *Ido1* DMR in placentas from control and BPA- and TBBPA-exposed mice were analyzed by bisulfite clonal sequencing ($N = 3$ dams per exposure group). (E) shows percentages of CpG methylation for each analyzed mouse. (F) shows bisulfite clonal sequencing data for placentas from control and BPA- and TBBPA-exposed mice (i.e., control, BPA, and TBBPA 1–3). Each circle represents a CpG site (labeled as sites 2, 3, 4, 5, and 7 that correspond to CpG sites 2–5 and 7 in the *Ido1* DMR). Methylated, filled circle; unmethylated, open circle. Each row represents an independent DNA strand. See numeric data in Table S7. Note: ANOVA, analysis of variance; BPA, bisphenol A; DMR, differentially methylated region; E, embryonic day; *Ido1*, indoleamine 2,3 deoxygenase 1; SEM, standard error of the mean; SNP, single nucleotide polymorphism; TBBPA, tetrabromobisphenol A.

10–25 μg/kg BW per day TBBPA, however, resulted in fetal death in rats.[45] The present study, and that of Haneke 2002,[45] suggest that TBBPA exposure below the oral reference dose of reproductive toxicity adversely affects fetal survival in rodents.

One novel finding of the present study was that BPA- and TBBPA-induced effects on pregnancy loss were associated with an altered maternal immune cell environment in mice. Pregnancy loss is associated with lower numbers of maternal Tregs in mouse spleen[120] and thymus[73] and human peripheral blood[66,70,71,72,121] and decidua.[66,70,71,72] Tregs have been identified as potent suppressors of inflammatory effector T cells that may elicit an attack on the semiallogeneic fetus, promoting pregnancy complications or loss of the pregnancy.[64] The Treg population increases as pregnancy advances, a process that is partly driven by activation of the aryl hydrocarbon receptor (AHR) by kynurenine metabolites in naïve $CD4^+$ T cells. AHR activation was associated with higher mRNA expression of *Foxp3*, the master transcriptional regulator gene for Tregs, and promotion of Treg differentiation in mice.[63,122] Fewer $CD4^+$ T cells could be indicative of fewer lymphocytes available to differentiate to Tregs, although further analysis of other T helper cell subtypes, including Th1 and Th2 cells,[123,124] would provide more information as to whether BPA and TBBPA specifically target Tregs. In addition, studies that explore AHR-mediated Treg generation using *Ahr* null or conditional knockout (KO) mice would further elucidate the causative link between lower Treg number and fetal resorption in BPA- and TBBPA-exposed mice.

Aluvihare et al. showed that depletion of Tregs, denoted as $CD25^+$ lymphocytes, induced pregnancy failure in allogeneic pregnancies but not in syngeneic mouse pregnancies.[80] The observation was consistent with results presented in this study showing that allogeneic mouse pregnancies are more susceptible to pregnancy loss relative to syngeneic pregnancies when challenged by factors that reduce Tregs (in this case BPA and TBBPA). One potential mechanism is that mouse fetal alloantigens drive a significantly larger increase in Tregs during early gestation in allogeneic pregnancies to reduce alloreactivity to paternal antigens.[83] Lower Treg numbers in the BPA- and TBBPA-exposed allogeneic mouse pregnancies potentially reflect a challenge to maternal–fetal immune tolerance in limiting responses to paternally inherited alloantigens. In contrast to Zhao et al. 2007,[83] however, the present study reports that allogeneic pregnancies had lower Tregs compared with syngeneic; the discrepancy could be related to differences in mouse strains used in the studies. Furthermore, one limitation of the study was the use of the B6XB6 mating combination (selected to correlate better with the molecular studies of *Ido1*) instead of CBA females mated to CBA males as the syngeneic pregnancy model. Therefore, the distinct effects on pregnancy loss could be partially linked to the inherent strain-specific differences in B6 vs. CBA females. In general, the present study supports a role of Tregs in maintaining successful allogeneic mouse pregnancies that are more similar to the semiallogeneic fetus in human pregnancy.

Disruptions in IDO1-mediated tryptophan–kynurenine catabolism, which drives Treg expansion in healthy pregnancies, has been proposed as a mechanism for pregnancy loss in humans[67,125,126] and animals.[68,127,128] Immunohistochemistry and RT-qPCR analysis has shown that placentas from women with recurrent spontaneous abortion had lower IDO1 protein and mRNA levels compared with those from normal pregnancies.[67] Overexpression and upregulation of *Ido1* in the placenta and decidua using a recombinant lentivirus and CTLA4Ig gene transfer, respectively, have improved embryonic absorption rates in the CBAXDBA/2 recurrent pregnancy loss mouse model and was associated with elevated levels of peripheral Tregs.[74,75] The present study was limited by the lack of genetic models to demonstrate the ability of *Ido1* overexpression in trophoblast giant cells to rescue BPA- and TBBPA-induced pregnancy loss. Furthermore, although a more accurate method for cell-specific quantification of *Ido1* mRNA in isolated trophoblast giant cells would be informative (e.g., fluorescence-activated cell sorting), no cell surface marker specific for trophoblast giant cells currently exists. Despite the limitations, the present study observations suggest that proper dosage of IDO1 expression contributes to pregnancy maintenance only in allogeneic mouse pregnancies. The conclusion is supported by the fact that fecundity and litter size were not affected in syngeneic IDO1 KO pregnancies, although the pregnant mice developed multiple preeclampsia phenotypes.[129] Future use of allogeneic *Ido1* KO pregnancies and genetic tools for *Ido1* overexpression would further elucidate the causative links among IDO1, exposure, and Tregs in mice.

One postulated mechanism regulating proper dosage of placental *Ido1* expression was through DNA methylation-dependent genomic imprinting in mice.[88] Imprinted genes play a critical role in development, and disruptions to these genes by environmental exposures have been linked to fetal growth restriction in mice[130] and humans,[131] defective development of the mouse[77,132] and human placenta,[133] and increased susceptibilities to metabolic disorder in mice[134,135,136] and reproductive disease in humans.[137] Human[138] and animal studies[77,97,139,140] have shown that exposure to BPA leads to epigenetic dysregulation of imprinted genes; however, this study is the first to show that TBBPA can modulate DNA methylation of imprinted genes in mice. The contribution of *Ido1* epigenetic regulation in pregnancy was recently reported in placentas from the abortion-prone CBAXDBA/2 mouse pregnancy model.[88] CBAXDBA/2 placentas exhibited ∼20% higher mean DNA methylation than controls at CpG sites 4 and 7 at the *Ido1* DMR;[88] these epigenetic changes correlated with 20–30% higher rates of fetal loss.[141] The present study on placentas from BPA- and TBBPA-exposed mice shows a similar correlation between the degree of DNA methylation changes at CpG sites 4 and 7 at the *Ido1* DMR and pregnancy loss (i.e., ∼5–10% higher DNA methylation and ∼10–15% higher rates of fetal resorption relative to controls). Importantly, elevated *Ido1* methylation levels observed in these pregnancy loss-prone mice translated to humans given that the putative *IDO1* DMR was hypermethylated in a subset of placentas obtained from human first trimester miscarriages.[88] The present investigation suggests that the *Ido1* gene is susceptible to BPA and TBBPA exposure and that its epigenetic dysregulation influences pregnancy success. Studies to further elucidate epigenetic regulatory mechanisms related to *Ido1* may provide insights into its role in pregnancy maintenance. For example, using DNA methyltransferase 1 (*Dnmt1*) KO mice could elucidate how global loss of methylation as a result of reduced expression of the maintenance DNA methylation machinery in BPA- and TBBPA-exposed mice influences *Ido1* expression and pregnancy outcomes.

BPA exposure induces male-specific effects in glucose tolerance,[136,142] insulin resistance,[136,142] and pancreatic beta cell growth[142,143] in mice. Studies have suggested that male-specific effects are linked to differential DNA methylation reprogramming of imprinted genes in males and females.[144] BPA-induced effects in insulin secretion and glucose tolerance, for example, were observed in male rodents and associated with DNA hypermethylation of the imprinted *Igf2* gene.[134,136,145] In the present report, BPA and TBBPA exposure was associated with a higher percentage of *Ido1* DMR DNA methylation in male and female mouse placentas, respectively, suggesting that there were potentially sex-specific differences in DNA methylation programming depending upon the chemical. Alternatively, many studies have shown sex

differences in the capacity to metabolize the parent compound of BPA,[13,16,146,147] and higher concentrations of BPA have been found in male fetuses of human pregnancies.[12] These studies suggest that another explanation could be that there are sexually dimorphic differences in metabolizing BPA and TBBPA.

In conclusion, the research presented here shows that maternal exposure to BPA and TBBPA adversely affect pregnancy maintenance in allogeneic mouse pregnancies and suggest shared mechanisms that involve altered maternal–fetal immune tolerance through lower Treg number and expression of IDO1. These studies warrant future investigation to establish causative links between *Ido1* expression and maternal Treg populations. Studies that incorporate adoptive transfer of splenic Tregs will determine whether higher Tregs rescue pregnancy loss in mice exposed to BPA and TBBPA as demonstrated in abortion-prone mouse models.[73,148]

Acknowledgments

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